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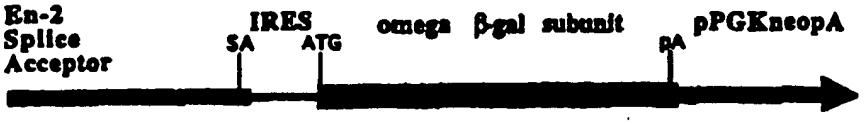
(54) Title: COMPLEMENTATION TRAP



(57) Abstract

Methods and DNA constructs are provided for detection and manipulation of a target eucaryotic gene whose expression is restricted to certain tissues or specialized cell types. The methods include transforming a cell with a first indicator component under the control of a promoter selected for its restricted expression in a particular cell or tissue. The cell is also transformed with a gene trap vector encoding a second indicator component. The cell is allowed to differentiate to produce a specialized cell or tissue which is monitored for expression of both the first and second indicator components, thereby detecting a gene into which the trap vector has integrated which is expressed in the same cell or tissue type as the selected promoter.

COMPLEMENTATION TRAP

Field of the Invention

The present invention relates to gene entrapment vectors and their use in gene discovery, and their use in screening for or making cells and organisms that are mutated for such genes. This invention also relates to the use of such entrapment vectors to identify tissue specific transcription control elements such as promoters and enhancers and for generating transgenic animals displaying restricted expression of transgenes. This invention also relates to trap vectors comprising a splice acceptor and a sequence encoding a reporter gene.

Background of the Invention

Genomic based drug discovery is largely dependent upon the identification of specific genomic targets. Thus, cloning, sequencing, and identification of function of mammalian genes is a first priority in a genomic based drug discovery. In particular, it is important to identify and make use of genes which are spacially and/or temporally regulated in the organism.

Animal model systems such as the fruit fly and the worm are often used in gene identification because of ease of manipulation of the genome and ability screen for mutants. While these systems have their limitations, large numbers of developmental mutations have been identified in those organisms either by monitoring the phenotypic effects of mutations or by screening for expression of reporter genes incorporated into developmentally regulated genes.

Many features of the mouse make it the best animal model system to study gene function. However, the mouse has not been used for large scale classical genetic mutational analysis because random mutational screening and

analysis is very cumbersome and expensive due to long generation times and maintenance costs.

A disadvantage in using animal models for the identification of genes is the need to establish a transgenic animal line for each mutational event. This disadvantage is alleviated in part by using embryonic stem (ES) cell lines because mutational events may be screened *in vitro* prior to generating an animal. ES cells are totipotent cells isolated from the inner cell mass of the blastocyst. Methods are well known for obtaining ES cells, incorporating genetic material into ES cells, and promotion of differentiation of ES cells. ES cells may be caused to differentiate *in vitro* or the cells may be incorporated into a developing blastocyst in which the ES cells will contribute to all differentiated tissues of the resulting animal. Vectors for transforming ES cells and suitable genes for use as reporters and selectors are also well known.

Gene entrapment strategies have been employed to identify developmentally regulated genes. One type of entrapment vector is called a "promoter trap", which consists of a reporter gene sequence lacking a promoter. Its integration is detected when the reporter is integrated "in-frame" into an exon. "Gene trap vectors" target the much more prevalent introns of the eucaryotic genome. The latter vectors consist of a splice-acceptor site upstream from a reporter gene. Integration of the reporter into an intron results in a fusion transcript containing RNA from the endogenous gene and from the reporter gene sequence.

Gene trap vectors may be made more efficient by incorporation of an internal ribosomal entry site (IRES) such as that derived from the 5' non-translated region of encephalomyocarditis virus (EMCV). Placement of a IRES

site between the splice acceptor and the reporter gene of a gene trap vector means that reporter gene product need not be translated as a fusion product with the endogenous gene product, thereby increasing the likelihood that integration of the vector will result in expression of the reporter gene product.

Examples from the literature of the use of promoter and gene trap vectors as well as such vectors including an IRES sequence, are listed below. Some examples involve the identification of developmentally regulated or tissue specific events making use of ES cell lines.

1. Canadian Patent application no. 2,166,850 (open for public inspection July 11, 1996) Vectors and the Use Thereof for Capturing Target Genes: describes the use of transmembrane sequence encoding gene trap vectors to isolate and identify secretory proteins. Also see United States Patent No. 5,767,336 issued June 16, 1998.

2. US Patent 5,364,783 issued Nov. 15, 1994. Retrovirus Promoter Trap Vectors: describes retroviral vectors that are used to isolate transcriptionally active chromosomal regions and to identify promoter sequences. The reporter gene is placed in the U3 or U5 control region of the retrovirus.

2. Gossler, A., et al. (1989). Mouse Embryonic Stem Cells and Reporter Constructs to Detect Developmentally Regulated Genes. Science 244:463-465: describes the use of enhancer trap gene trap vectors for use in identifying developmentally regulated genes. The gene trap vector consists of the mouse En-2 splice acceptor upstream from lacZ (reporter) and a selector gene (hBa-neo).

Summary of Invention

This invention makes use of known genes whose expression is restricted to specific tissue, tissues or specialized cells ("restricted expression") to facilitate identification and manipulation of new genes and their associated transcription control elements which have similar patterns of expression.

Accordingly, this invention provides a method of detecting a target gene having restricted expression in a eucaryotic organism, which comprises the steps of:

(i) transforming a eucaryotic cell with a DNA sequence encoding a first indicator component under the control of a promoter having restricted expression;

(ii) transforming the cell of (i) or a descendent of the cell of step (i), by operably integrating into the cell's genome DNA lacking a promoter but which comprises a sequence encoding a second indicator component;

(iii) producing tissue or specialized cells from the cell of (ii); and

(iv) monitoring the tissue or specialized cells of (iii) for a detectable indicator resulting from both the first and second indicator components.

This invention also provides a method of obtaining a gene, a part of a gene, transcription control element or other nucleotide sequence, having restricted expression which includes isolating endogenous DNA flanking of the sequence encoding the second indicator component from a

cell or descendent of a cell prepared as described above in which the detectable indicator is found. This invention also provides a method of modifying the gene, transcriptional control element or other nucleotide sequence obtained as described above which includes modification of the endogenous DNA which flanks the coding sequence of the second indicator component.

This invention also provides a method of providing a eucaryotic organism producing a detectable indicator in a specialized cell or tissue of the organism which includes growing a multi-cellular eucaryotic organism from a cell or cell descendent from a cell prepared as described above, in which the detectable indicator is found. The organism may also express in the specialized cell or tissue, the product of heterologous DNA expressed with DNA flanking of the second indicator component of the detectable indicator.

This invention makes use of a gene entrapment strategy whereby a detectable indictor is detected when more than one component of the indicator is expressed in the same cell. One method of this invention involves stable and operable insertion of a nucleic acid construct which includes one indicator component of the complementation trap under the control of a known promoter having restricted expression into the genome of the cell. This is followed by further transformation of the cell, or a cell derived from the previously transformed cell, by insertion into the cell's genome of a trap vector which includes a DNA which encodes a second indicator component. Insertion of the trap vector into an endogenous gene may result in the transcription of both the endogenous gene and the second indicator component under control of the endogenous promoter and subsequent translation of the second component. If the trap vector integrates into an endogenous gene which is expressed in the same cell or

tissue type as the first indicator component, a indicator resulting from the combination of the first and second indicator components may be detected. Thus, this invention allows one to distinguish target eukaryotic genes which are expressed in the same cell or tissue type as the promoter controlling the first component of the entrapment system. This system, as embodied by this invention is termed a "complementation trap".

This invention provides a DNA construct comprising a splice acceptor upstream of a sequence encoding a component of a detectable indicator according to this invention. The component may be, for example, an enzyme fragment or subunit, or an enzyme functional in a pathway leading to production of a detectable indicator. This invention also provides a DNA construct comprising a promoter having restricted expression upstream of a sequence encoding a component of a detectable indicator as described above.

This invention also provides a first DNA construct comprising a splice acceptor upstream of a sequence encoding a first peptide selected from the group consisting of an alpha peptide and an omega peptide of β-galactosidase (β-gal), wherein the first peptide lacks β-gal activity but is capable of alpha complementation to produce active β-gal. This invention also provides a eucaryotic cell and a non-human, multi-cellular eucaryotic organism having operably and stably incorporated into its genome, the first DNA construct described above. This invention also provides a kit including the first DNA construct and, a second DNA construct comprising a promoter having restricted expression upstream of a sequence encoding a second β-gal peptide selected such that the second peptide is capable of alpha complementation with the first peptide. This invention also provides a eucaryotic cell and a

non-human, multi-cellular eucaryotic organism having operably and stably incorporated into its genome the first DNA construct and which also expresses the second DNA construct.

Trap vectors useful for this invention, including the above-described first DNA construct, may also contain additional elements such as an internal ribosome entry sequence (IRES) upstream of the second indicator component (eg. the first peptide) or, sequences that allow for directional integration of a heterologous gene, such as a recognition site for site specific recombination (eg. lox).

This invention may be used to identify tissue or cell type specific genes. For example, a nucleic acid construct containing the coding sequence of a first indicator component under control of a known tissue specific promoter is "seeded" into cells which may be made to differentiate or whose nuclear material may be incorporated into cells which are capable of differentiation. The second component is then introduced into a cell having the "seeded" construct, by means of the trap vector. The resulting cells, or cells derived from those cells (eg. progeny), are then induced to differentiate (for example by addition or withdrawal of a chemical inducer/repressor) or allowed to spontaneously differentiate. The cells are then screened for activity of the indicator which will occur in the cell or tissue type in which the promoter is functional.

This invention is particularly useful for screening cell or tissue specific genes or transcriptional control elements such as promoters and enhancers, in any animal from which embryonic stem (ES) cell lines may be obtained. The ES cell is subjected to the complementation trap process described above. The ES cells are then allowed to differentiate *in vitro* and cells are screened for the

indicator. ES cells demonstrating expression of the indicator may be introduced into a blastocyst to produce an animal which will exhibit the same pattern of expression. Where introduction of the trap vector results in disruption of the target gene, the animal resulting from the mutated ES cells will be useful as a "knock-out" animal useful for study of the target gene. Alternatively, location of the trap vector into the endogenous gene will provide means for subsequent disruption of the target gene for production of "knock-out" animals. The trap vector of this invention may be used as a means for locating, cloning, sequencing, and further mutation of the target gene or promoter or enhancer sequences associated with the target gene.

This invention will make new transcriptional control elements available for use in making new transgenic animals displaying cell, tissue or organelle specific expression of transgenes. Promoters made available by use of this invention may themselves be made to control the first indicator component in the complementation trap of this invention in a "leap-frog" procedure whereby new genes having the same restriction pattern as the promoter or may be located.

Brief Description of Drawings

Figure 1: is a schematic illustrating a DNA construct useful in this invention comprising the myeloid cell specific promoter CD11b controlling the sequence encoding a β-galactosidase alpha peptide terminated by the human growth hormone poly-adenylation signal. Downstream is a selection cassette, which in this case confers resistance to hygromycin driven by the phosphoglycerate kinase promoter.

WE CLAIM:

1. A method of detecting a target gene having restricted expression in a eucaryotic organism, which comprises the steps of:

(i) transforming a eucaryotic cell with a DNA sequence encoding a first indicator component under the control of a promoter having restricted expression;

(ii) transforming the cell of (i) or a descendent of the cell by operably integrating into the cell's genome, DNA lacking a promoter but which comprises a sequence encoding a second indicator component;

(iii) producing tissue or specialized cells from the cell of (ii); and

(iv) monitoring the tissue or specialized cells of (iii) for a detectable indicator resulting from both the first and second indicator components.

2. The method claim 1 wherein the eucaryotic cell is an ES cell.

3. The method of claims 1 and 2 wherein the first and second indicator components are inactive fragments or subunits of an enzyme which, when combined, provide an active enzyme detectable by its activity.

4. The method of claim 1 or 2 wherein the first and second indicator components are independently detectable or selectable, and the detectable indicator is the presence of both indicator components in a cell.

5. The method of claim 1 or 2 wherein the first and second indicator components react in a sequence of reactions which result in a detectable indicator.

6. The method of any one of claims 1-5 which comprises the additional step of isolating DNA endogenous to the eucaryotic cell which flanks integrated DNA comprising the second indicator component.

7. A method of producing an eucaryotic organism comprising a detectable indicator associated with a target gene having restricted expression, comprising steps (i) and (ii) of claim 1 to produce a transformed cell, followed by the step of growing an eucaryotic organism from said transformed cell.

8. The method of claim 7 wherein the eucaryotic organism is a non-human mammal and the transformed cell is an ES cell.

9. A DNA construct comprising, in a 5′ to 3′ direction, a splice acceptor and a sequence encoding an inactive subunit or fragment of an enzyme, wherein said subunit or fragment is active when combined with a further subunit or fragment of an enzyme.

10. The DNA construct of claim 9 wherein the sequence encodes an alpha or omega fragment of β-galactosidase.

11. The DNA construct of claim 9 or 10 further comprising an IRES.

12. The combination of:

(i) a DNA construct for integration into the genome of an eucaryotic cell comprising a sequence encoding a first indicator component under the control of a promoter having restricted expression; and

(ii) A DNA construct for integration into the genome of a eucaryotic cell, comprising in the 5′ to 3′ direction, a splice acceptor and a sequence encoding a second

indicator component and an optional IRES, wherein expression of both the first and second indicator components in said cell is detectable.

13. A eucaryotic cells transformed by the combination of DNA constructs of claim 12.

14. The cell of claim 13 which is an ES cell.

INTERNATIONAL SEARCH REPORT

Inter :al Application No
PCT/CA 98/00677

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/85 A01K67/027 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KAMAL CHOWDHURY ET AL.: "Evidence for the stochastic integration of gene trap vectors into the mouse germ line" NUCLEIC ACIDS RESEARCH, vol. 25, no. 8, 15 April 1997, pages 1531-1536, XP002079742 OXFORD GB cited in the application see abstract see page 1531, right-hand column, paragraph 2 - page 1532, right-hand column, last paragraph --- -/--	1-14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 October 1998	16/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WILLIWM C. SKARNES ET AL.: "A gene trap approach in mouse embryonic stem cells: the lacZ reporter is activated by splicing, reflects endogenous gene expression, and is mutagenic in mice" GENES & DEVELOPMENT, vol. 6, June 1992, pages 903-918, XP000569059 cited in the application see the whole document	1-14
A	SUZAN DZIENNIS ET AL.: "The CD11b promoter directs high-level expression of reporter genes in macrophages in transgenic mice" BLOOD, vol. 85, no. 2, 15 January 1995, pages 319-329, XP002079743 cited in the application see the whole document	1-14
A	EP 0 514 173 A (MICROGENICS CORPORATION) 19 November 1992 see page 2, line 50 - page 3, line 47 see page 4, line 8 - line 20	
P,X	WO 98 24918 A (HÖLZER, DIETER) 11 June 1998 see page 1, paragraph 1 - paragraph 3 see page 4, paragraph 4 - page 10, paragraph 1	1,2,5-8

1

INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/CA 98/ 00677

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 7 and 8 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat Application No
PCT/CA 98/00677

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 514173 A	19-11-1992	AT 162891 T	15-02-1998
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